

Direct, Simplified, and Sensitive Assay of Angiotensin II in Plasma Extracts Performed with a High-affinity Monoclonal Antibody

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A very simple, fast, and sensitive RIA of angiotensin (Ang) II has been developed, based on a monoclonal antibody with high affinity and specificity, making possible the direct measurement of circulating Ang II in human plasma after solid-phase extraction. The purified monoclonal antibody 4D8 has an association constant of 1.3×10^{11} L/mol with Ang II and a cross-reactivity of <1% for Ang I. The assay can detect as little as 0.8 fmol of Ang II in 2 mL of plasma and is not influenced by the presence of Ang I. Analytical recoveries between 112% and 116% were obtained for Ang II added to human plasma at physiological concentrations. Comparison of the RIA with a reversed-phase, high-performance liquid chromatographic method followed by RIA to measure Ang II in human plasma samples from normal and hypertensive subjects—and from normotensive subjects before and after an acute inhibition of angiotensin-converting enzyme with captopril (50 mg)—showed a high degree of correlation ($r^2 = 0.93$) between the two methods.

Additional Keyphrases: hypertension • radioimmunoassay • HPLC compared • captopril • angiotensin-converting enzyme

The octapeptide angiotensin (Ang) II is the major hormone of the renin-angiotensin system; it regulates blood pressure and aldosterone secretion.⁶ A sensitive and specific RIA is required for measuring Ang II in human plasma because the concentration of this analyte is low and its precursor, Ang I, is structurally similar.

Monoclonal antibodies (MAbs) are routinely used in clinical diagnosis and are replacing conventional antisera because of their availability and their greater specificity and affinity. Of the MAbs against Ang II that have been described (1-5), several are specific, but most have low affinity for Ang II.

Here we report on the production and characterization of a potent MAb (4D8) directed against Ang II and used in the development of an RIA for measuring immunoreactive Ang II in human plasma in the presence of Ang I.

Materials and Methods

Materials

Source of peptides. International standard Ang II (lot 86/538) was kindly provided by the National Institute for Biological Standards and Control (NIBSC; Herts., UK). Ang I, Ang II, Ang III, and Ang(1-7) were purchased from Bachem (Torrance, CA); Ang(3-8), Ang(4-8), Ang(5-8), Ang(2-10) from Peninsula Labs (Saint-Helena, Merseyside, UK); tetradecapeptide (human form) saralasin and [Sar¹]Ang II from Sigma Chemical Co. (St. Louis, MO). All other peptides were synthesized in our laboratories.

Other reagents. Bovine serum albumin (BSA), bovine gamma globulin, and hemocyanin (from *Limulus polyphemus* hemolymph) were obtained from Sigma Chemical Co. Acetonitrile, polyethylene glycol (PEG) 6000, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from Merck (Darmstadt, FRG). Amerlex M (a sheep anti-mouse IgG coupled to magnetic particles) and ¹²⁵I-labeled-Ang II ([¹²⁵I]Ang II) were purchased from Amersham (Bucks., UK). Polystyrene Star tubes were from Nunc (Roskilde, Denmark). Triethylamine was obtained from Prolabo (Paris, France).

Production of MAbs

Preparation of immunogen. Ang II was conjugated to hemocyanin with a water-soluble carbodiimide, as described by Freedlander and Goodfriend (6). The protein content of the final product was determined by the method of Lowry et al. (7), and the number of residues of Ang II incorporated was estimated by adding [¹²⁵I]Ang II to the coupling reaction. The results indicated that 400 Ang II residues were conjugated to 1 hemocyanin molecule (molecular mass 1740 kDa).

Immunization procedure. A high-responder Biozzi mouse (8) and a BALB/c mouse were immunized with 100 µg of the hemocyanin-Ang II conjugate. The first injections were given subcutaneously in complete Freund's adjuvant; the final injection was given intravenously 3 days before the fusion under conditions designed to prevent anaphylactic shock (9).

Production of hybridomas. P3-X63-Ag8-653 murine myeloma cells were fused with immune spleen cells by a procedure previously described (10). Briefly, 2×10^8 splenocytes and 4×10^7 myeloma cells were fused in the presence of 1 mL of 400 g/L PEG 1540 solution (Riedel de Haën, Seelze, Hannover, FRG). At the end of the fusion procedure, the cells were distributed (0.2 mL/well) at a density of 150×10^3 cells/well into microculture plates containing macrophages as feeder cells ($5 \times$

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⁶ Nonstandard abbreviations: Ang, angiotensin; MAb, monoclonal antibody; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PEG, polyethylene glycol; [¹²⁵I]Ang, [¹²⁵I]-labeled angiotensin; and ACE, angiotensin-converting enzyme.

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10⁵ BALB/c peritoneal exudate cells/well). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium. Anti-Ang II MAbs in culture supernatants were detected by their binding to [¹²⁵I]Ang II (see next section). Antibody-producing hybridomas were immediately cloned by the limiting dilution method.

Immune serum testing and MAb screening procedure. Anti-Ang II antibodies in the mouse serum and in culture supernate were detected by their binding to [¹²⁵I]Ang II. We incubated 100 μ L of each culture supernate or dilution of mouse serum overnight at 4 °C with 100 μ L of [¹²⁵I]Ang II (~10⁴ counts/min) in 0.1 mol/L imidazole · HCl buffer, pH 7.5, containing BSA, 2 g/L (RIA buffer). The separation of bound from free Ang II was performed with 1 mL of a 200 g/L PEG 6000 solution as previously described (11). The radioactivity in the bound fraction was determined in the pellet after centrifugation.

Mass production and purification of MAbs. Ascites were produced after intraperitoneal injection of cloned selected hybridoma cells into pristane-treated BALB/c mice. MAbs were then purified from the ascites by affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Ey et al. (12).

Characterization of MAbs: specificity and affinity. Antibody specificity was evaluated by RIA. The RIA was performed with purified MAb in the presence of various concentrations of Ang II (standard curve) or Ang II-related peptides. Briefly, 100 μ L of MAb at a dilution that binds 80% of tracer was incubated in RIA buffer overnight at 4 °C with 50 μ L of Ang II or Ang II-related peptide and 50 μ L of [¹²⁵I]Ang II as tracer. Separation of bound from free Ang II was performed with PEG 6000 or 0.5 mL of Amerlex M magnetized antibodies. The cross-reactivities were calculated by the method of Abraham (13). The association constant of the purified anti-Ang II MAb was determined by Scatchard analysis (14) of the Ang II inhibition curves.

Subjects

Group I consisted of 21 normal healthy ambulant volunteers. Group II comprised nine normal subjects who received a single oral dose of 50 mg of captopril; blood samples were collected from their antecubital veins before (in the upright and the supine position separately) and 60 and 120 min after drug administration. Group III comprised 12 hypertensive patients, whose blood was collected in the supine position without inhibition of angiotensin-converting enzyme (ACE). Blood samples from Group I were used to determine normal concentrations of Ang II by the direct RIA with MAbs. All plasma samples from Groups II and III were used to determine Ang II concentrations by the direct RIA and by the comparison method, reversed-phase high-performance liquid chromatography (HPLC) followed by RIA.

Procedures

Blood collection and solid-phase extraction. Plasma blood samples were collected in tubes kept on ice at 4 °C and containing EDTA, 6.25 mmol/L, the renin inhibitor

SR 43845, 10 nmol/L (15), and the ACE inhibitor MK422, 10 μ mol/L (final concentrations), to prevent generation as well as degradation of Ang II (16). The samples were immediately centrifuged (700 \times g) at 4 °C and the resulting plasma was stored at -70 °C until analyzed. Using Bond Elut pH cartridges (Analytichem, Harbor City, CA), we extracted angiotensin peptides from 2 mL of plasma (17), as follows: For each plasma sample, one cartridge containing 100 mg of phenylsilyl-silica was prewashed with 1 mL of methanol, followed by 1 mL of water. We passed 2 mL of cold plasma through the cartridge and then washed this with 1 mL of water. Adsorbed angiotensin peptides were eluted from the cartridge with 0.5 mL of methanol and collected in a conical polypropylene tube previously coated with BSA (5 g/L in 0.1 mol/L imidazole · HCl buffer, pH 7.5, containing NaN₃, 0.2 g/L) overnight at room temperature, or in a borosilicate glass tube. The methanol was evaporated under reduced pressure in a centrifuge evaporator. Plasma Ang II in the solid-phase extract was then measured by our direct RIA method with immobilized MAb (detection of immunoreactive Ang II) or after separation from other angiotensins by HPLC followed by RIA (detection of authentic Ang II).

Direct RIA. The immobilized MAb was prepared as follows: 300 μ L of a solution of purified goat anti-mouse IgG secondary antibody at a concentration of 5 mg/L in PBS (phosphate-buffered saline) Dulbecco (Seromed, Biochrom, Berlin, FRG) was incubated at room temperature overnight in polystyrene Star tubes. The next day, the tubes were washed with PBS, filled with 1 mL of PBS + BSA (0.3 g/L), incubated for 30 min at 37 °C, and washed again; then, 250 μ L of a solution of MAb 4D8 (in a concentration that binds 30% of tracer) was added and the tubes were incubated at 4 °C overnight. After washing, the tubes were used without further delay.

To assay immunoreactive Ang II in plasma, we dissolved the solid-phase extract in 500 μ L of RIA buffer, then added 200 μ L of extract or Ang II (0.6 to 80 fmol per assay) to 50 μ L of [¹²⁵I]Ang II tracer (4000 counts/min). After incubating this overnight at 4 °C, we separated free Ang II from antibody-bound Ang II by adding 1 mL of RIA buffer to the tubes and aspirating. The [¹²⁵I]Ang II bound to the tubes was measured with a gamma counter.

HPLC coupled to RIA. Plasma Ang(1-8) octapeptide (authentic Ang II) was measured by using the same monoclonal antibody after separating the various angiotensin peptides by HPLC, according to a previously described method (18) with minor modifications. After reconstituting the solid-phase extract in 140 μ L of 0.1 mol/L acetic acid, we vortex-mixed the sample and injected 100 μ L of this solution into the HPLC. Angiotensins were separated by using a 100 \times 4.6 mm column (packed with 3- μ m particle size Hypersil ODS; SFCC, Neuilly Plaisance, France) connected to a pump system (Waters 501; Millipore, Bedford, MA) fitted with an automatic injector (Waters 712 WISP). Elution was at 45 °C with an isocratic mobile phase of acetonitrile and 86 mmol/L triethylammonium phosphate (pH 3.0)

(17/83 by vol) and at a flow rate of 0.6 mL/min. Based on the retention times of angiotensin peptide standards, Ang II was collected at 7.28 min after injection, in four to eight 105- μ L fractions into tubes containing 50 μ L of 0.1 mol/L imidazole-HCl buffer, pH 7.5, containing BSA, 9 g/L. We then added to each fraction 100 μ L of [125 I]Ang II tracer (3000 counts/min) and 100 μ L of a solution of MAb 4D8 (binding 30% of tracer) in RIA buffer. Ang II standards (0.2 to 50 fmol in 50 μ L of RIA buffer) and 105 μ L of mobile phase were likewise incubated with 100 μ L of antibody and 100 μ L of tracer. After a 72-h incubation at 4 $^{\circ}$ C, free Ang II was separated from antibody-bound Ang II by adding to each sample 250 μ L of a charcoal-dextran suspension (11 g of charcoal and 1 g of dextran per liter of RIA buffer) and incubating for 10 min at room temperature. After centrifugation at 3000 \times g for 20 min, the supernate was aspirated, and the radioactivity of the charcoal pellets (containing free [125 I]Ang II) was measured in a gamma counter. The individual values of each of the four to eight fractions of each sample were added together and the sum was compared with the value obtained by direct RIA measurement.

Statistics. Concentrations of Ang II in the normotensive subjects are expressed as means \pm SE. Correlation coefficients of linear regression were calculated by using the least-squares method.

Results

Although the immunization protocols were the same, the serum titer of the antibodies from the BALB/c mouse (1/20 000) was markedly greater than that obtained in the Biozzi mouse (1/20). Fusion with spleen cells from the Biozzi mouse (F141) yielded 20 anti-Ang II hybridomas, whereas 140 anti-Ang II hybridomas were obtained from the fusion performed with the spleen cells from the BALB/c mouse (F150).

MAb characterization. The most potent MAb produced by hybridoma from F141 had an association constant of 10^8 L/mol; in contrast, from fusion F150, we selected 12 MAbs with association constants $>10^{10}$ L/mol and 10 more between 10^9 and 10^{10} L/mol. MAb 4D8 was chosen for its high affinity, produced in ascites fluid, and purified; its association constant was 1.3×10^{11} L/mol.

MAb specificity. All of the MAbs tested showed comparable specificity profiles. Taking reactivity with Ang II as 100%, cross-reactivity was $<1\%$ for Ang I and $>100\%$ for Ang III. Cross-reactivity decreased with the successive loss of amino acids from the amino terminus for peptides generated by degradation of Ang II. No cross-reactivity was observed when peptides lacking phenylalanine in the carboxy terminus were tested (Figure 1). There was no cross-reactivity with the non-peptidic Ang II-receptor antagonists Dup 753 and CGP 42112A.

Characteristics of the direct RIA. In standard curves, 10 fmol of Ang II displaced 50% of the tracer in 10 separate assays (CV 6%). The least quantity of peptide that significantly displaced tracer (2 SD from the point for zero peptide on the standard curve) was obtained by

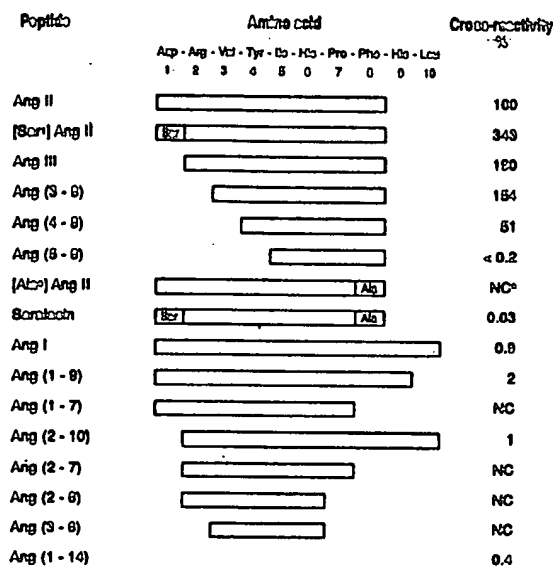


Fig. 1. Cross-reactivity of MAb 4D8 with peptides related to Ang II. Cross-reactivity values for peptides were calculated by using the equation: $100 \times \text{fmol of Ang II required to inhibit 50\% binding of } [^{125}\text{I}]\text{Ang II to antibody/fmol of peptide required to inhibit 50\% binding}$. NC: No detectable cross-reactivity ($<0.01\%$). There was also no cross-reactivity with Dup 753, CGP 42112A, SR 43845, or MK 422 (not shown).

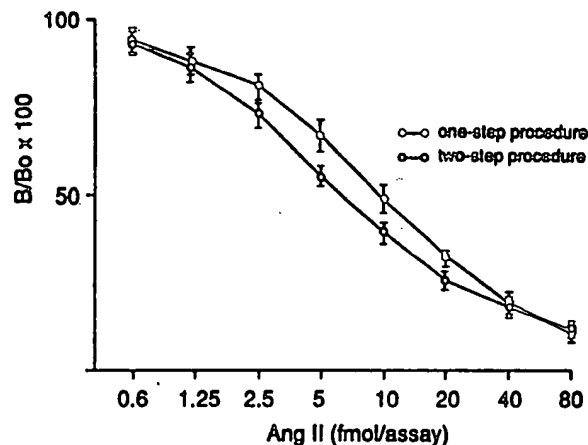


Fig. 2. Standard curves for RIA with immobilized MAb 4D8: comparison between a one-step procedure (24-h incubation at 4 $^{\circ}$ C) and a two-step procedure (24-h incubation at 4 $^{\circ}$ C before adding [125 I]Ang II, followed by a second 24-h incubation).

adding 0.85 fmol of unlabeled Ang II. In a control assay, we measured a concentration of Ang II <1 pmol/L in pre-extracted plasma. When the immobilized MAb used was preincubated for 24 h with either the plasma extract or the Ang II standard, 7 fmol of Ang II displaced 50% of the tracer (CV 6%) (Figure 2).

Analytical recovery and normal values. Increasing amounts of Ang II were added to a plasma sample before extraction. The recovery values were between 112% and 116% (Table 1).

Plasma immunoreactive Ang II was measured in samples taken from 21 normal healthy ambulant volunteers, ages 20 to 40 years (Group 1). The mean concentration was 5.2 (SE 2.8) pmol/L.

The CV for within-assay precision (10%) was estab-

Table 1. Analytical Recovery Experiment

Ang II, pmol/L		
Added	Recovered	Recovery, %
2.5	2.9	116
5.0	5.7	114
10.0	11.2	112

Original plasma samples contained Ang II at 3.5 pmol/L. Recovery was calculated by direct RIA after extraction.

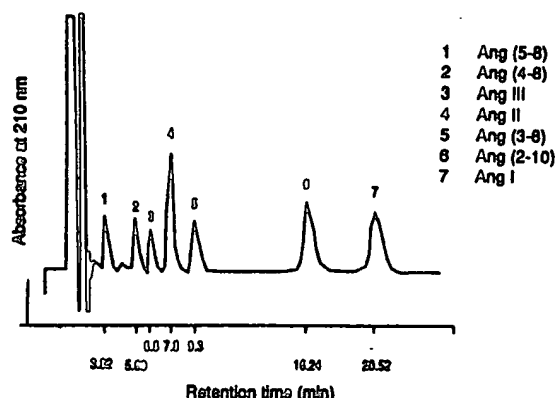


Fig. 3. HPLC profile of angiotensin peptides. 50–150 ng of standard angiotensin peptides was injected and detected by ultraviolet absorbance at 210 nm.

lished by assaying in the same RIA eight aliquots of a single plasma containing Ang II at 2.7 pmol/L. The CV for between-assay precision (9%) was determined by measuring the Ang II content of a single plasma sample (mean 4.1 pmol/L) on 5 different days.

Comparison with HPLC coupled to RIA method. To validate the direct RIA, we made separate measurements of immunoreactive and authentic (HPLC) Ang II in plasma from subjects with widely different plasma concentrations of the peptides. Thirty-nine blood samples were taken from nine normal subjects in the upright and supine positions and also at 60 and 120 min after acute ACE inhibition (with 50 mg of captopril) in the supine position. Blood samples also were collected from 12 hypertensive patients in the supine position. After solid-phase extraction, plasma Ang II concentrations were determined by the present RIA method and by the previously established method (HPLC linked to RIA). Figure 3 shows that the angiotensin peptides were completely resolved by HPLC before the RIA. As depicted in Figure 4, Ang II concentrations in 39 human plasma samples measured by both methods were nearly identical over the range of concentrations from 0.4 to 52.2 pmol/L and correlation was excellent ($y = 0.98x + 0.38$, $r^2 = 0.93$, $SEE = 2.4$). Plasma concentrations of immunoreactive Ang II did not exceed the concentrations of authentic Ang II, even in subjects given ACE inhibitor. At peak ACE inhibition (60 min after captopril), neither immunoreactive nor authentic Ang II was detectable.

Discussion

This very sensitive and specific RIA for measuring Ang II was developed with the use of a very potent MAb

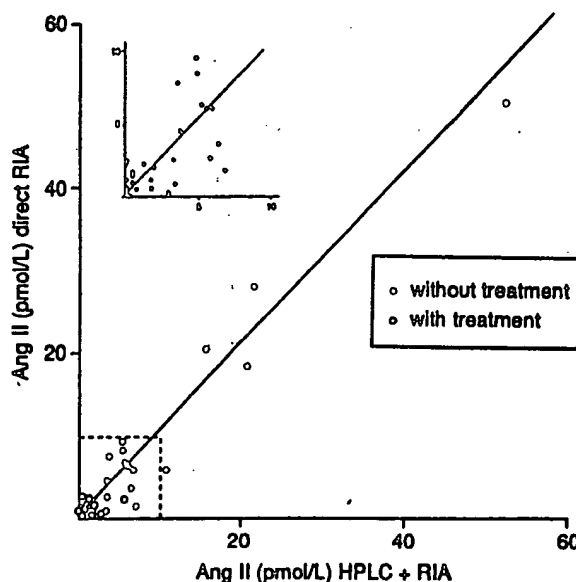


Fig. 4. Correlation between Ang II concentration in plasma from subjects in Groups II and III as determined by direct RIA and by HPLC followed by RIA.
 $y = 0.93x + 0.38$, $SEE = 2.4$, $r^2 = 0.93$, $n = 39$ (see Materials and Methods).

(4D8). The anti-Ang II antiserum titer of the mouse whose spleen cells were used for the fusion that led to hybridoma F150-4D8 was very high, even taking into account the homology between human and murine Ang II. Indeed, the association constant (1.3×10^{11} L/mol) was unusually high for an anti-hapten MAb (19). In the standard curve of the RIA developed by Nussberger et al. (5) with an anti-Ang II MAb, 32 fmol of Ang II was needed to displace half of the tracer. In the standard curve of the RIA we developed with the MAb 4D8, 10 fmol displaced half of the tracer. The latter assay is sensitive enough to measure immunoreactive Ang II in human plasma. The reproducibility of the assay at the mid-dose concentration, however, could be further increased by preincubating immobilized MAb with the Ang II-containing sample: under these conditions, 7 fmol displaced half of the tracer. Values of Ang II in plasma from healthy volunteers were in the same range as that reported by others (16, 17, 20–23).

The low cross-reactivity with Ang I might be explained by the fact that MAb 4D8 has carboxy-terminal specificity. Furthermore, there was little or no cross-reactivity with peptides for which the C-terminal amino acid was not phenylalanine. MAb 4D8 therefore should be especially valuable for determining Ang II concentrations in patients treated with inhibitors of ACE or renin.

All the MAbs obtained with our Ang II-hemocyanin conjugate had the same specificity. Apparently, aspartic acid is not in the epitope recognized by these antibodies; in addition, the charge on this amino acid may have a negative effect on the binding, as shown by the better recognition of Ang III and [Sar¹]Ang II. The cross-reactivities of MAb 4D8 with Ang II-related peptides decreased with the successive removal of the N-terminal

amino acid, suggesting that Arg, Val, and Tyr are involved in the epitope. Taken together, our results suggest that MAb 4D8 recognizes a discontinuous epitope involving Arg, Val, and Tyr at the amino terminus and the phenylalanine at the carboxy terminus.

The potent nonpeptidic antagonists for tissue receptors of Ang II, Dup 753 (24, 25) and CGP 42112A (26), which specifically block the Ang II receptors in adrenal, liver, or uterus at nanomolar concentrations, showed no cross-reactivity with MAb 4D8. These results suggest that MAb 4D8 and the Ang II receptor recognize Ang II differently.

Using MAb 4D8, we measured plasma immunoreactive Ang II in a group of subjects having a wide range of concentrations of circulating renin and Ang II, including normal volunteers before and after acute ACE inhibition with captopril. When ACE is acutely inhibited, a high concentration of renin and hence Ang I builds up in the circulation, whereas the plasma concentration of Ang II falls to very low values. Concentrations of immunoreactive Ang II were not higher than the concentrations of authentic Ang II in these subjects, showing that the direct RIA did not result in a falsely high estimate of Ang II concentrations in the plasma of these subjects. Indeed, interference from Ang I was negligible: MAb 4D8 showed low cross-reactivity with Ang I (<1%). Although MAb 4D8 cross-reacts with the N-terminal heptapeptide, hexapeptide, and pentapeptide fragments of Ang II, our results can be explained by the fact that concentrations of Ang III and other immunoreactive metabolites in human plasma are low (20, 22, 27, 28); in addition, the in vitro degradation and generation of cross-reacting material was successfully blocked. Earlier observations by Nussberger et al. (16) suggested that use of a renin inhibitor during blood sampling prevents in vitro generation of Ang I and Ang II, as well as the formation of angiotensin metabolites, and thereby renders the values obtained by direct measurement of immunoreactive Ang II closer to the concentrations of authentic Ang II.

In conclusion, the present RIA method for measuring Ang II in human blood is accurate and does not require separating Ang II from its immunoreactive metabolites. This fast and sensitive direct RIA for Ang II, based on an MAb with high affinity and specificity, allows easy routine measurement of immunoreactive Ang II in human plasma.

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